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AN EXAMINATION OF A PUTATIVE SYSTEM FOR THE IN VITRO
SYNTHESIS OF DROSOPHILA TRYPTOPHAN PYRROLASE

by

John P. Phillips

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Zoology

Approved:

Major Professor

Head of the Department

Dean of Graduate Studies

UTAH STATE UNIVERSITY
Logan, Utah

1967

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John P. Phillips

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ABSTRACT

An Examination of a Putative System for the in vitro
Synthesis of *Drosophila* Tryptophan Pyrrolase

by

John P. Phillips, Master of Science

Utah State University, 1967

Major Professor: Dr. John R. Simmons
Department: Zoology

A system, reported by Morrison (1964), for the in vitro synthesis of *Drosophila* tryptophan pyrrolase has been critically studied. This putative system is based on the addition of a *Drosophila* "RNA" extract to a *Drosophila* cell-free protein synthesizing system; the synthesized enzyme is then allowed to catalyze a specific reaction, the product of which is measured by direct adsorption.

The results of this study present a three-fold argument against the interpretation of synthesis of tryptophan pyrrolase in the Morrison in vitro system: (1) RNA from a null-allele, v^{36f} , stimulated the same tryptophan pyrrolase "activity" in the in vitro system as Canton-s RNA. (2) The Bratton-Marshall test, a valid kynurenine assay in this system, detects no tryptophan pyrrolase activity in systems showing "activity" when assayed by the direct adsorption method used by Morrison. (3) An ascorbate dependent, nonenzymatic reaction, which produced a pseudotryptophan pyrrolase activity, was detected in the Morrison assay system. This pseudoactivity, which is eliminated by the substitution of 2-mercaptoethanol for ascorbate, was interpreted by Morrison as being representative of tryptophan pyrrolase produced in the in vitro system.

(43 pages)

INTRODUCTION

In studying the mechanism of action of the suppressor of vermilion in *Drosophila*, it seemed advantageous to have a method for the in vitro synthesis of tryptophan pyrrolase, the enzyme controlled by the vermilion locus. A report of such a technique exists in the literature (Morrison, 1964). In reviewing this report several criticisms arose, including (1) lack of proper controls; (2) the use of an ascorbate containing enzyme incubation system which is now known to produce a nonenzymatic side reaction; and, (3) the use of an ambiguous direct adsorption assay for kynurenine. A re-examination of the procedures, results, and interpretations reported by Morrison seemed necessary before the technique could be used reliably and was the major objective of this study.

REVIEW OF LITERATURE

Genetics of the Vermilion and Suppressor of Vermilion Loci

The sex-linked, recessive vermilion (v, 1-33.0+) eye color mutants are phenotypically distinguished by a lack of brown eye pigments. Green (1952) genetically distinguished two series of vermilion mutants by their differential interaction with a nonallelic X chromosome mutant, suppressor of vermilion (su-v, 1-0.0+). Several su-v mutants have been reported, but all of them are allelic and functionally indistinguishable. The v mutants of one series (v^s) are suppressed by su-v and consequently approach wild type in the production of ommochromes. Mutants of the other series (v^u) are not suppressed by su-v and remain indistinguishable from v. Green (1954) reported crossing over between v¹, a v^s allele, and v^{36f}, a v^u allele, indicating that the two series of v mutants have representatives which are pseudoallelic to each other. Pseudoallelism at the vermilion locus was also studied by Barrish and Fox (1956). They found that extracts from three different v mutants acted to produce three different antibodies when injected into rabbits. This would be expected if the v mutants are pseudoallelic, and each produces an immunologically distinct protein. The suppressible alleles also differ from the unsuppressible alleles in that some brown pigment is produced by the v^s alleles under starvation conditions.

The interactions between the v alleles and su-v have yet to be explained adequately. Using the extent of brown pigmentation as the

criterion, Baglioni (1960) suggested that suppression is dependent upon a homozygous condition for su-v. However, su-v homozygosity does not seem to be an absolute requirement for suppression since $\frac{\text{su-v}}{\text{su-v}^+} \frac{v^s}{v^s}$ flies have slightly darker eyes than $\frac{v^s}{v^s}$ flies. Heterozygous vermilion alleles in combination with homozygous suppressor $\frac{\text{su-v}}{\text{su-v}} \frac{v^u}{v^s}$ give rise to flies with eyes darker than those with $\frac{\text{su-v}}{\text{su-v}} \frac{v^u}{v^u}$ genotype but lighter than those with $\frac{\text{su-v}}{\text{su-v}} \frac{v^s}{v^s}$ genotype. Shapard (1960) has observed that a deficiency, T(1:2)Bld, for the tip of the X chromosome, including the su-v locus, when in heterozygous combination with a su-v allele produces the same phenotypic effect as the su-v homozygote. Obviously, these results must be substantiated by quantitative pigment determination of these genotypes before any reliable conclusions can be drawn. This could be done by extraction and spectrophotometric determination of pigment content or by enzymatic assay of the tryptophan pyrrolase activities of the genotypes.

Biochemistry of the Vermilion Locus

The biosynthesis of ommochromes in *Drosophila* from tryptophan has been studied, and an incomplete pathway has been constructed (Fig 1). Several mutants have been described which constitute genetic blocks at different steps in this pathway.

Green (1949) found that the block in ommochrome production by the vermilion mutants is accompanied by an accumulation of non-protein tryptophan. The su-v mutants caused the reappearance of the brown pigment and a parallel decrease in the amount of free tryptophan. Subsequently, Green (1952) showed that the v mutants are able to convert kynurenine or formyl-kynurenine to brown pigment. Thus, it appeared that the v mutants constitute genetic blocks in the ommochrome pathway prior to the formation of formyl-kynurenine. Early attempts to find the enzyme responsible for the conversion of tryptophan to formyl-kynurenine were largely unsuccessful. However, in 1959, Baglioni, using a modification of the highly sensitive Bratton-Marshall test for kynurenine, showed that flies homozygous for a v mutant lack the enzyme which catalyzes the first step in the metabolic degradation of tryptophan. He later showed (Baglioni, 1960) that this enzyme is tryptophan pyrrolase and reported that suppressed-vermilion flies $\frac{\text{su-v}}{\text{su-v}} \frac{\text{v}^{\text{S}}}{\text{v}^{\text{S}}}$ possess 30% of the wild type tryptophan pyrrolase activity. The v^S mutants $\frac{\text{su-v}^{+}}{\text{su-v}^{+}} \frac{\text{v}^{\text{S}}}{\text{v}^{\text{S}}}$ are characterized by having less than 15% wild type activity. The effect of su-v on restoration of tryptophan pyrrolase activity has also been reported by Kaufman (1962) who found 15% and 1-2% respectively and by Marzluf (1965a, 1965b) who reported values of 7% and less than 1% for tryptophan pyrrolase activities of suppressed-vermilion and v^S flies

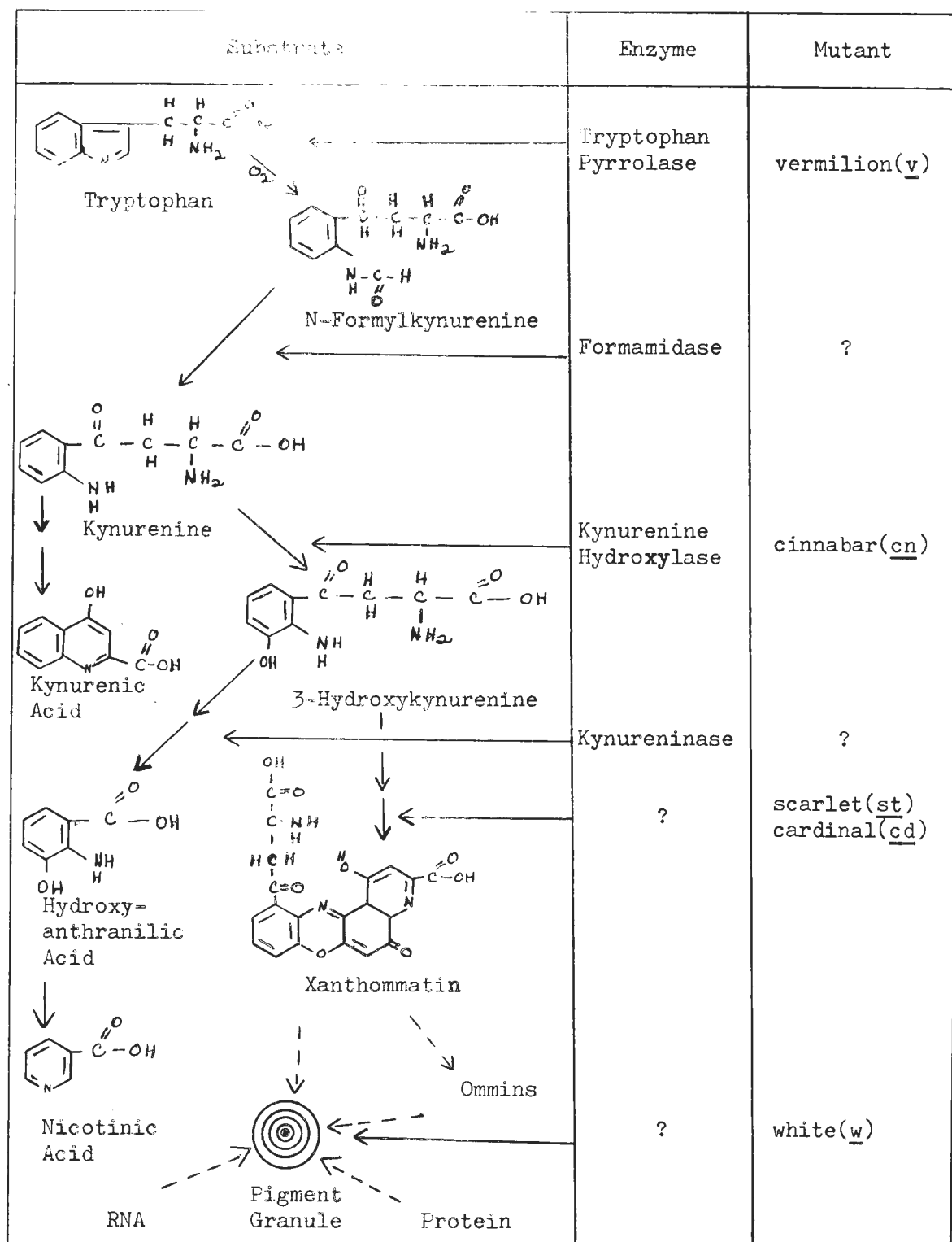


Figure 1. Postulated Pathway of Ommochrome Synthesis in *Drosophila* (cf., Ziegler, 1961; Wagner & Mitchell, 1964)

respectively. The discrepancies in these results are probably due to many factors, the most notable of which is the progressive development of more sensitive enzyme assays. Marzluf (1965a, 1965b) has shown by kinetic behavior, pH optima, heat inactivation, inhibition by Cu^{++} , and energy of activation, that the enzyme from suppressed-vermilion flies is indistinguishable from the wild type enzyme.

Tryptophan Pyrrolase

One major pathway in the metabolic utilization of tryptophan is initiated by the enzyme tryptophan pyrrolase (Fig 1). This reaction was first studied in a rat liver system by Knox and Mehler (1950). Their initial studies suggested a two-step mechanism involving a substrate peroxidation followed by an oxidation. However, later work (Knox, 1959; Tanaka & Knox, 1959) showed that the reaction involves only the oxidative fission of the indole ring of tryptophan yielding formyl-kynurenine and hydrogen peroxide, which maintains the enzyme in the reduced or ferrous state. By IEC nomenclature, tryptophan pyrrolase is referred to as tryptophan oxygenase, (1.13.1.12). A comparison of the properties of tryptophan pyrrolase isolated from *Drosophila*, rat liver, and *Pseudomonas* is given in Table 1. Attempts have been made to purify the enzyme from all three sources but they have been relatively unsuccessful (Marzluf, 1965a; Tanaka & Knox, 1959; greengard & Fidgeison, 1969).

TABLE 1

Comparison of Properties of Tryptophan Pyrrolase from
Drosophila, *Pseudomonas*, and Rat Liver
 (from Marzluf, 1964)

Comparison	<i>Drosophila</i>	Rat Liver	<i>Pseudomonas</i>
K_m	$1 \times 10^{-3}M$	$4 \times 10^{-4}M$	$3.5 \times 10^{-4}M$
specific for L-tryptophan	yes	yes	yes
sensitive to dialysis	yes	yes	no
heme prosthetic group	probably	yes	yes
heme group bound tightly	yes	no	yes
substrate inducible	yes	yes	yes
substrate stabilizes	no	yes	no
pH optimum	7.4	7-7.2	7-7.2
reduction required for activity	yes	yes	yes
precipitates at pH 5.4	no	yes	?
ammonium sulfate saturation required for precipitation	43-57%	30-50%	0-40%
inhibition by Cu^{++} , NaN_3 , and NH_2OH	yes	yes	yes

Other Mutants in the Ommochrome Pathway

Kynurenine formamidase, the enzyme controlling the hydrolysis of formyl-kynurenine to kynurenine, has been demonstrated to be present in all *Drosophila* stocks tested, including a few which lack the ability to synthesize brown pigment (Glassman, 1956). Since no mutants have been found which lack kynurenine formamidase, no studies of the genetic control of this enzyme have been done.

The conversion of kynurenine to 3-hydroxykynurenine is blocked by the mutant, cinnabar (cn, 2-57.5). Since no enzymatic studies have been done on this enzyme in *Drosophila*, it is only assumed that this block results in the alteration or elimination of kynurenine hydroxylase activity. Hayaishi (1962) has studied this enzyme from a rat liver source. It is interesting that this enzyme appears particle bound since it sediments with the mitochondrial fraction during centrifugation.

The mutants scarlet (st, 3-44.0) and cardinal (cd, 3-75.7) are phenotypically indistinguishable from vermilion and have been found to accumulate 3-hydroxykynurenine. These mutants have been tentatively assigned to the control of enzymes catalyzing the condensation of 3-hydroxykynurenine to form xanthommatin (Wagner & Mitchel, 1964; Ziegler, 1961).

The mutants of the white locus (w, 1-1.5), which also block ommochrome synthesis, are unusual in that they concomitantly result in the absence of the red eye pigments. On this basis, it has been postulated that the white mutants act at a point or points which are common to both synthetic pathways. Also, there is some indication that the white locus controls in some way the structure or composition of the pigment

granules upon which the terminal reactions of both synthetic pathways may be located (Beadle, 1945; Ziegler, 1961).

In vitro Protein Synthesis

Studies of intermediary metabolism have established that most physiological reactions are enzyme catalyzed. Investigations performed to determine the relationships between enzyme-catalyzed reactions and the hereditary material of the cell have shown that such reactions are under strict genetic control. Some of the basic concepts for our present understanding of gene-enzyme relationships were formulated by Garrod in the early 1900's (Garrod, 1923) and further developed by studies with eye color mutants of *Drosophila* (Beadle, 1945). These studies, as well as the famous investigations with *Neurospora crassa* by Beadle and Tatum (1941), led to the description of numerous biosynthetic pathways. More importantly, from a genetic standpoint they offered confirmation of a basic relationship among genes, biochemical reactions, and the enzymes catalyzing the reactions. Mutations were shown to lead to the loss of ability to perform single biochemical reactions, and this loss was associated with the absence of particular enzyme activities. It is evident from these studies that there is a specific genetic region or gene concerned with the presence of each active enzyme. Later, inactive proteins were detected in mutants (Suskind, et al., 1955); and the nature of the change in the structure of many altered proteins was established, in each case involving the substitution of a single amino acid (Ingram, 1963; Yanofsky, et al., 1964). With protein structure information of this type and with the proper genetic systems,

fine structure genetic maps could be prepared and the positions of the mutant sites compared with the positions within the corresponding protein at which the amino acid changes occurred. Mutational studies have then established that the linear genetic map is a representation of a corresponding structure, the polypeptide chain.

Another technique has been developed in the last few years which allowed even further study of gene-enzyme relationships. This is in vitro, RNA-dependent protein synthesis. Most of the initial work with this procedure was based on non-specific synthesis of proteins whose formation depends upon the presence of free amino acids, an energy generating system, tRNA, ribosomes, natural or synthetic mRNA, and the enzymes, cofactors, etc., found in the soluble fraction of cell-free extracts (eg., Nirenberg & Matthaei, 1961). The formation of synthetic mRNA's has allowed the observation that different amino acids are incorporated into protein-like material, depending on the nucleotide composition of the mRNA (Nirenberg & Jones, 1963; Ochoa, 1963). These findings, as well as the demonstration of binding of specific tRNA's to ribosome-trinucleotide complexes (Nirenberg & Leder, 1964; Leder & Nirenberg, 1964), have made it possible to establish the nucleotide sequences of most coding units.

The in vitro synthesis of specific, biologically active proteins in response to naturally occurring mRNA has been inherently more difficult to demonstrate. The ability of an E. coli, cell-free system to synthesize a TMV-like protein in response to TMV RNA was reported by Tsugita et al. (1962). This appears to be an unambiguous case of the response of a synthesizing system to a specific messenger in that the

messenger was foreign to the cell-free system. Two other reports claim to have induced the synthesis of specific enzymes by the appropriate messenger. Novelli and Eisenstadt (1963) attempt to show complete transcriptive and translative sequences in the in vitro production of E. coli B-galactosidase activity. Their conclusions are based on the DNase and the RNase sensitive appearance of B-galactosidase in a Gal⁻ cell-free system. Wainwright and McFarlane (1962) demonstrated the ability of a cell-free system from a Neurospora td mutant to produce tryptophan synthetase activity in the presence of wild type Neurospora RNA. These last two reports still leave some question about the de novo synthesis of the enzyme since the possibility of some type of activation or chain completion of a pre-existing mutant protein is not satisfactorily eliminated. Another recent report of in vitro enzyme synthesis in response to an exogeneous mRNA was given by Morrison (1964). He found that the addition of a wild type Drosophila RNA extract to a cell-free protein synthesizing system derived from a vermilion mutant stimulated what appeared to be tryptophan pyrrolase activity. However, the measurement of activity was based on a direct adsorption assay for kynurenine. That the "activity" was due to synthesized tryptophan pyrrolase was not satisfactorily established.

MATERIALS AND METHODS

Drosophila Stocks

The materials and methods used were those specified by Morrison (1964) with the exception of (1) *Drosophila* stocks used; (2) preparation of larvae; and (3) the mercaptoethanol enzyme incubation.

All *Drosophila* stocks were maintained at 25°C on standard medium, consisting of corn meal, agar, Brewer's yeast, sucrose, and propionic acid. Canton-s, a wild type strain was used as the standard reference for tryptophan pyrrolase activity. Two vermilion mutants were used as systems lacking tryptophan pyrrolase activity: v^1 , a leaky, suppressible mutant, and v^{36f} , a nonleaky, nonsuppressible mutant.

Larvae Preparation

Because of its active metabolic state, the third instar larval stage was chosen for use. Young adult flies were transferred to bottles containing a live yeast paste and were allowed to lay eggs for 24 hours, after which they were removed. Larval development was thus roughly synchronized. After development of the larvae to the third instar, they were separated from the yeast by vigorous shaking of the culture with warm tap water, allowing the yeast to become fully suspended. This larvae-yeast mixture was then poured over a #60 mesh screen to separate the larvae from the yeast suspension. The larvae were then washed several times in cold tap water, recollected on the screen, and used immediately.

RNA Extraction

Seventy-five g of freshly harvested larvae were suspended in 0.5 vol cold 0.6% sodium dodecyl sulfate (SDS) and 1.5 vol aqueous phenol and homogenized at 0-5°C in a large TenBroeck tissue grinder. The presence of detergent and phenol during the homogenization was to minimize the action of RNase. The homogenate was then extracted for 10 minutes by stirring at 0-5°C. This was followed by centrifugation of the homogenate at 2000xg for 10 minutes and removal of the aqueous layer. The interface was then combined with the phenol layer and the pellet and the mixture was re-extracted by stirring for 10 minutes at 45-50°C in an additional 0.5 vol of 0.6% SDS and 1.5 vol aqueous phenol. The aqueous layer was again removed after a 10 minute centrifugation at 2000xg and the extraction process was repeated, first at 65-70°C and then at 90-95°C. Each aqueous layer to be used was adjusted to 2% for potassium acetate. Precipitation of the "RNA" was then achieved by the addition of 2 vol cold 95% ethanol followed by storage at -20°C for 18-24 hours. After collection of the precipitate by centrifugation, it was washed several times with cold 95% ethanol, redissolved in 0.05M tris buffer, pH 7.8, dialyzed for 18-24 hours at 0-5°C against three changes of 0.05M tris, and stored in the dark at 0-5°C.

Preparation of the

Cell-free Protein Synthesizing System

Fifty g of \underline{v}^1 or \underline{v}^{36f} larvae were lightly homogenized with 3-6 strokes of a large clearance Dounce tissue grinder in an equal vol of homogenizing medium which contained 0.01M magnesium acetate, 0.1M potassium chloride, 0.01M 2-mercaptoethanol, 0.01M phenylthiourea,

and 0.05M tris, pH 7.8. The homogenate was filtered through 8 layers of cheese cloth and centrifuged at 15000xg for 15 minutes. The resulting supernatant was added to an equal vol of an incubation medium to give a final protein synthesizing system which contained, in uM/ml, magnesium acetate, 10; potassium chloride, 100; 2-mercaptoethanol, 2.5; phenylthiourea, 0.25; tris pH 7.8, 100; ATP, 1.0; GTP, 0.25; and creatine phosphate, 5.0. Creatine phosphokinase was added in sufficient amount to convert the creatine phosphate to creatine and ATP. The RNA was then added to a final concentration of 5.0 ug/ml and the system incubated at 37°C for 30 minutes. Control systems, containing an equal vol of tris in place of the RNA, were incubated simultaneously.

Preparation of Native Enzymes

Enzyme incubation. Two enzyme incubation systems were used. One incubation system, the one used by Morrison, was as follows: 1.0 ml aliquots of enzyme, either the native enzyme prep or the protein synthesizing system, were incubated at 37°C in a 5.0 ml total vol incubation system which contained, in final concentration, tryptophan, 6.6 uM/ml; sodium ascorbate, 11.0 uM/ml; hematin, 1.25 ug/ml; and sodium phosphate buffer, 110.0 uM/ml.

The second enzyme incubation system used was described by Kaufman (1962). This system, using 2-mercaptoethanol as the reducing agent, was as follows: 0.4 ml aliquots of the enzyme were incubated at 37°C in a 1.0 ml total vol incubation system which contained, in final concentration, tryptophan, 3.6 uM/ml; 2-mercaptoethanol, 100.0 uM/ml; and potassium phosphate buffer, pH 7.4, 0.75 uM/ml. At successive intervals

aliquots from the enzyme incubation systems were withdrawn and equally divided for use in two assay systems.

Assays. The first and simplest method of assay used in this study involved the direct spectrophotometric determination of kynurenine (Tanaka & Knox, 1959; Morrison, 1964). The second and most reliable method was a modification of the Bratton-Marshall test used by Kaufman (1962), which involves the diazotization of kynurenine to form a colored complex. The procedure was as follows: 1.0 ml samples of the incubation mixture containing either the protein synthesizing system or a native enzyme prep were withdrawn and placed in tubes containing 3.0 ml of 6.0% trichloroacetic acid. After allowing time for complete precipitation of the protein, the precipitate was removed by filtration. To 0.5 ml of the filtrate were added 0.2 ml N-(1-naphthyl)ethylenediamine dihydrochloride(0.1%) in that order, allowing three minutes between each addition for the completion of the reactions. The mixture was then allowed to develop for 18-24 hours in the dark, after which the O.D. at 560 mμ was measured. All steps were carried out at 0-5°C.

Nucleic Acid and Protein Determinations

Analysis of the nucleic acid and protein contents of the extracted "RNA" fractions and of the protein content of the enzyme preps were done by using the orcinol reaction for RNA determination (Schnieder, 1963), the diphenylamine reaction for DNA determination (Schnieder, 1963), and either the Biuret or the Folin reactions for protein determination (Layne, 1963).

EXPERIMENTAL RESULTS

Assay of Native Enzymes

The endogeneous tryptophan pyrrolase activities of the various *Drosophila* strains to be used for the cell-free protein synthesizing systems were determined (Figs 2, 3, and 4). An obvious requirement for any protein synthesizing system is that it must not possess any synthetic activity without the addition of the appropriate mRNA. The mutant v^{36f} was found to have the lowest tryptophan pyrrolase activity and was therefore chosen for use in all cell-free preparations. The tryptophan pyrrolase activity of Canton-s was used as the standard reference activity. The activities reported here are in accord with the previous findings (Baglioni, 1960; Kaufman, 1962; Marzluf, 1964).

Both assay systems used gave comparable results. The ratio of v^{36f} activity to Canton-s activity was constant between the two assays. A very interesting observation is the tryptophan dependent background "activity" present in the ascorbate incubation system. This background is positive in both assay systems, although it appears to be more sensitive to the direct adsorption assay. This tryptophan dependent background activity makes the interpretation of the no-tryptophan enzyme incubation controls rather difficult.

Assay of in vitro Synthesis

The capacities of the material extracted from Canton-s and v^{36f} to stimulate tryptophan pyrrolase synthesis in the cell-free system are

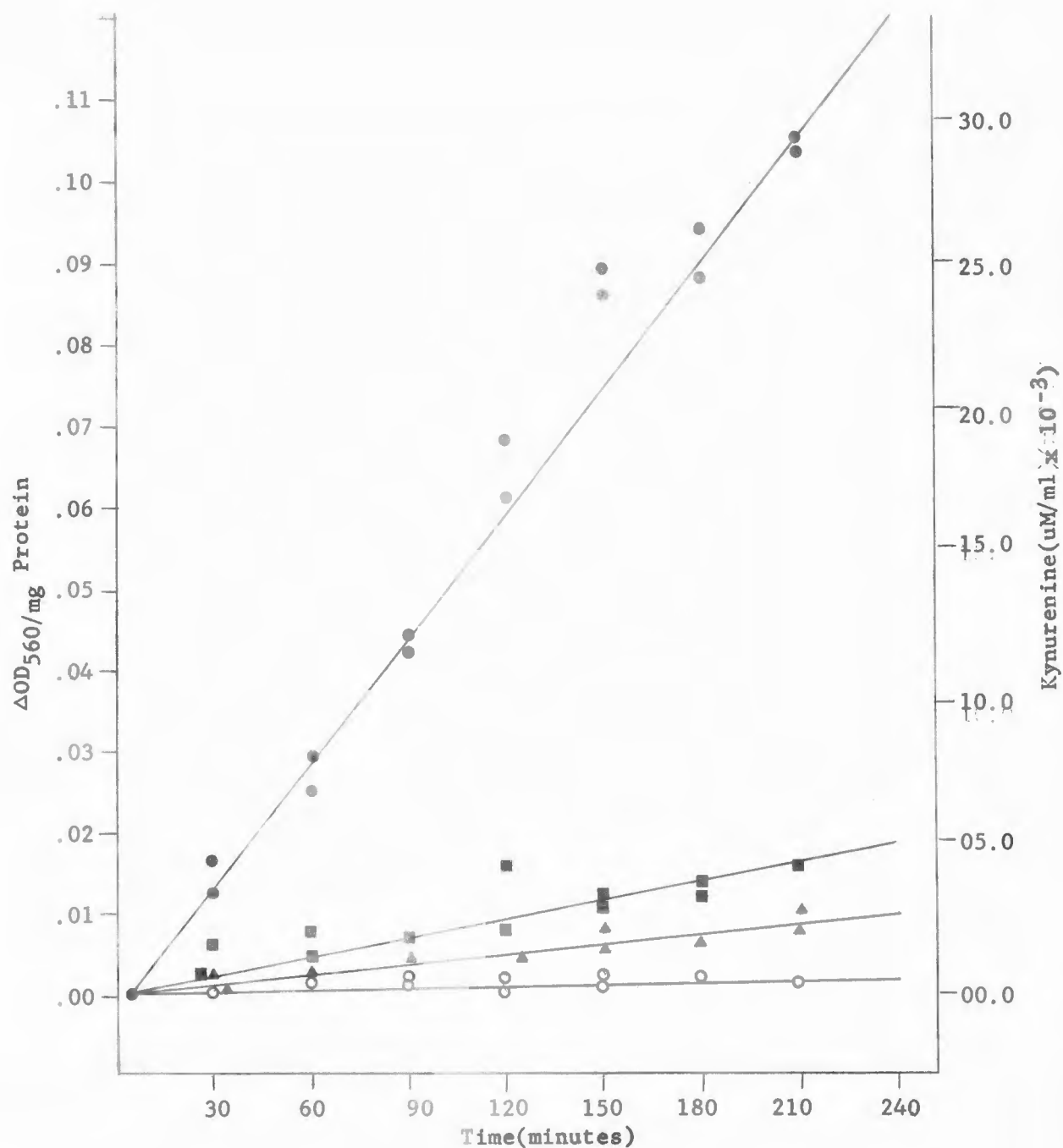


Figure 2. Assay of native enzymes: Mercaptoethanol incubation, Bratton-Marshall assay. (See Materials and Methods)

ENZYME		INCUBATION
(●-●)	Canton-s native	complete
(○-○)	Canton-s native	without tryptophan
(■-■)	v ¹	complete
(▲-▲)	v ^{36f}	complete

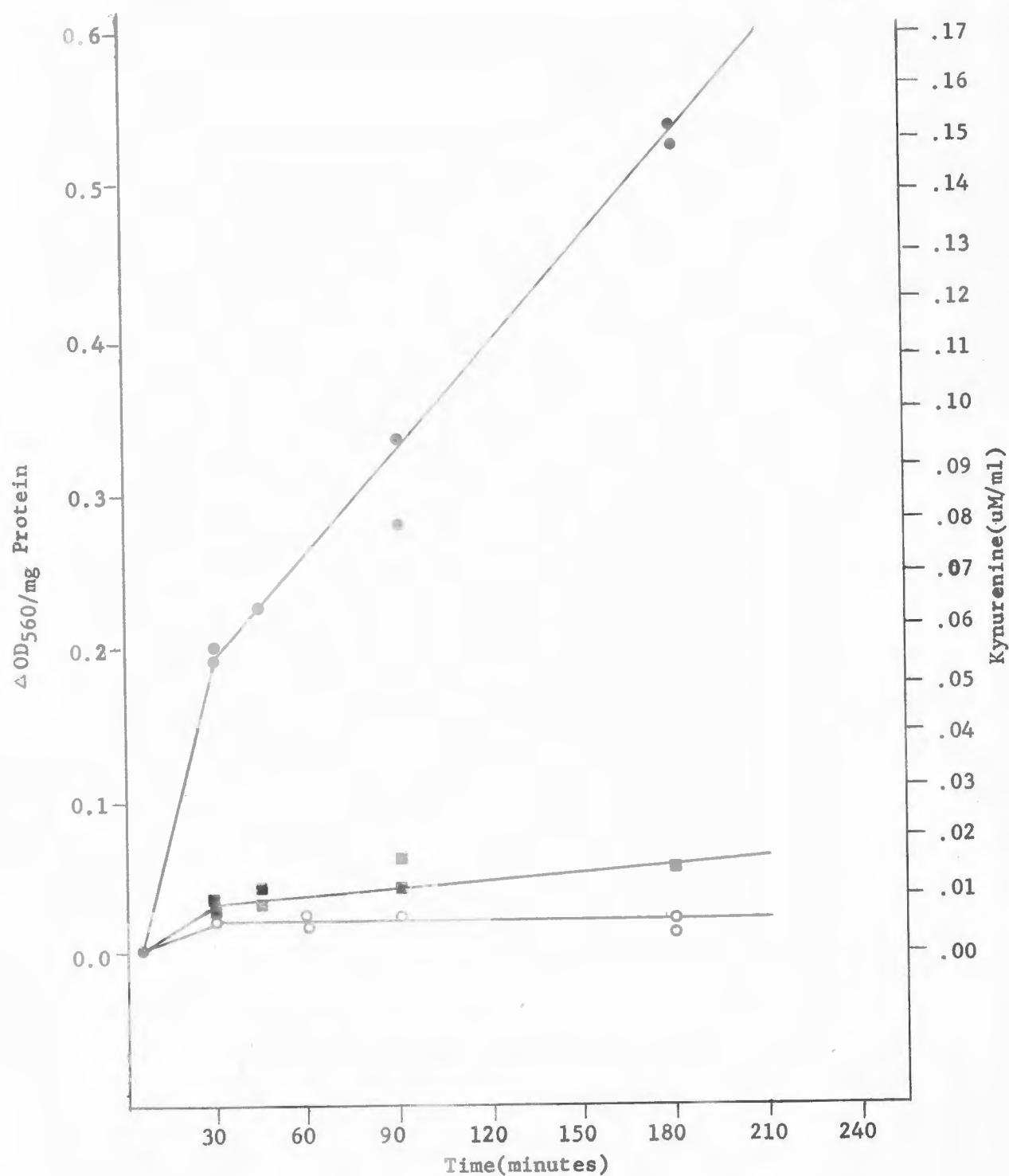


Figure 3. Assay of native enzymes: - Ascorbate incubation, Bratton-Marshall assay.

ENZYME		INCUBATION
(●-●)	Canton-s native	complete
(o-o)	Canton-s native	without tryptophan
(■-■)	y ^{36f} native	complete

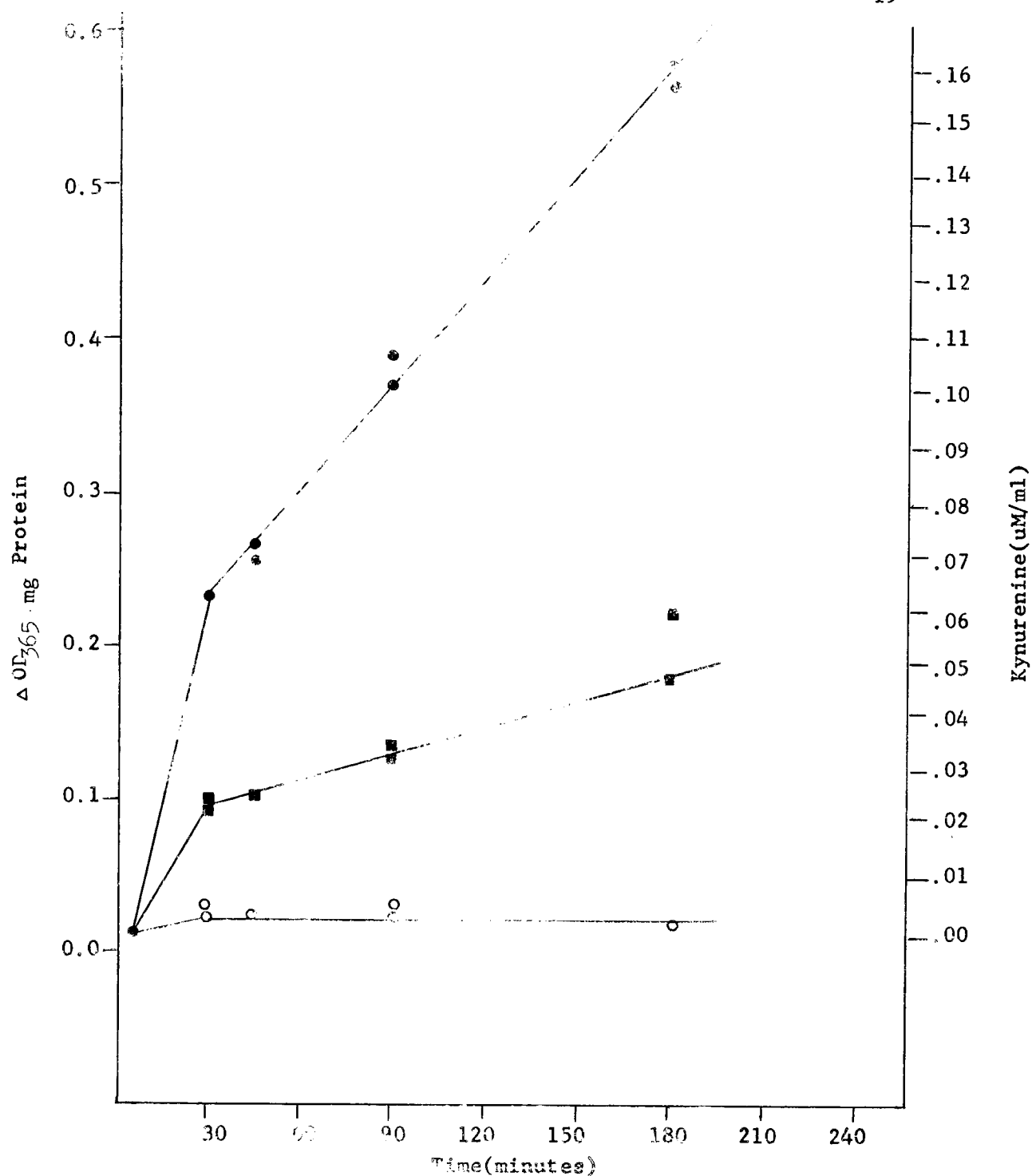


Figure 4. Assay of native enzymes: Ascorbate incubation, Direct adsorption assay

ENZYME		INCUBATION	
(●-●)	Canton-s native	complete	
(○-○)	Canton-s native	without tryptophan	
(■-■)	v ^{36f} native	complete	

shown in Figs 5, 6, and 7. The basic criteria for determining the validity of RNA-dependent tryptophan pyrrolase synthesis in the system are (1) the protein synthesizing system must show no tryptophan pyrrolase activity with the addition of the appropriate mRNA; (2) \underline{v}^{36f} RNA should not stimulate any tryptophan pyrrolase activity; (3) Canton-s RNA should stimulate tryptophan pyrrolase activity; and, (4) the Canton-s stimulated activity should be substrate (tryptophan) dependent. If one or more of the criteria are not satisfied using both assay procedures, then there is reason to suspect the validity of either the synthesizing system or the assay procedures.

Both the direct adsorption and the Bratton-Marshall assays show that the protein synthesizing system without any added RNA produces a significant amount of activity in the ascorbate incubation system. Thus, one immediately begins to suspect a fault in the system since \underline{v}^{36f} , the source of the cell-free system, has been shown to be completely lacking tryptophan pyrrolase activity. The second criterion, likewise, is not satisfied. The Bratton-Marshall assay shows no effect on tryptophan pyrrolase activity upon the addition of \underline{v}^{36f} RNA to the protein synthesizing system. However, assay of the same system by Morrison's direct adsorption method shows a positive increase in activity due to the addition of \underline{v}^{36f} RNA. The third criterion is not satisfied by either assay. Although the addition of Canton-s RNA to the protein synthesizing system does produce an activity over the no-RNA control as detected by the direct adsorption assay, the increase is the same as that produced by the addition of \underline{v}^{36f} RNA and, therefore, is not due to the synthesis of tryptophan pyrrolase. Assay by the

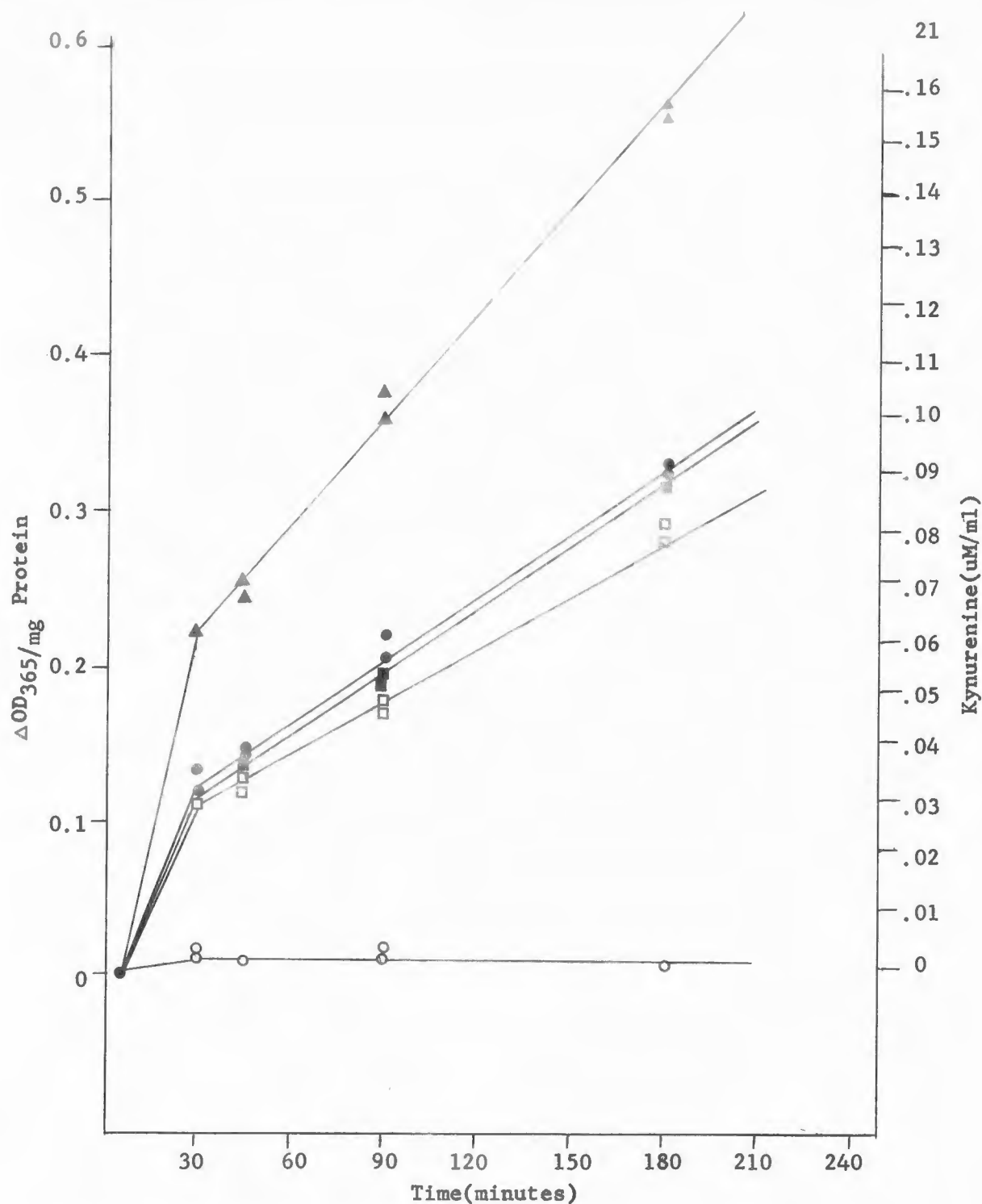


Figure 5. Stimulation of *in vitro* tryptophan pyrrolase synthesis by Canton-s and v^{36f} RNA: Ascorbate incubation, Direct adsorption assay.

ENZYME	INCUBATION
(●-●) PSS + Canton-s RNA	complete
(○-○) PSS + Canton-s RNA	without tryptophan
(□-□) PSS + tris buffer	complete
(■-■) PSS + v^{36f} RNA	complete
(▲-▲) Canton-s native	complete

Abbreviation: PSS = Protein Synthesizing System (see

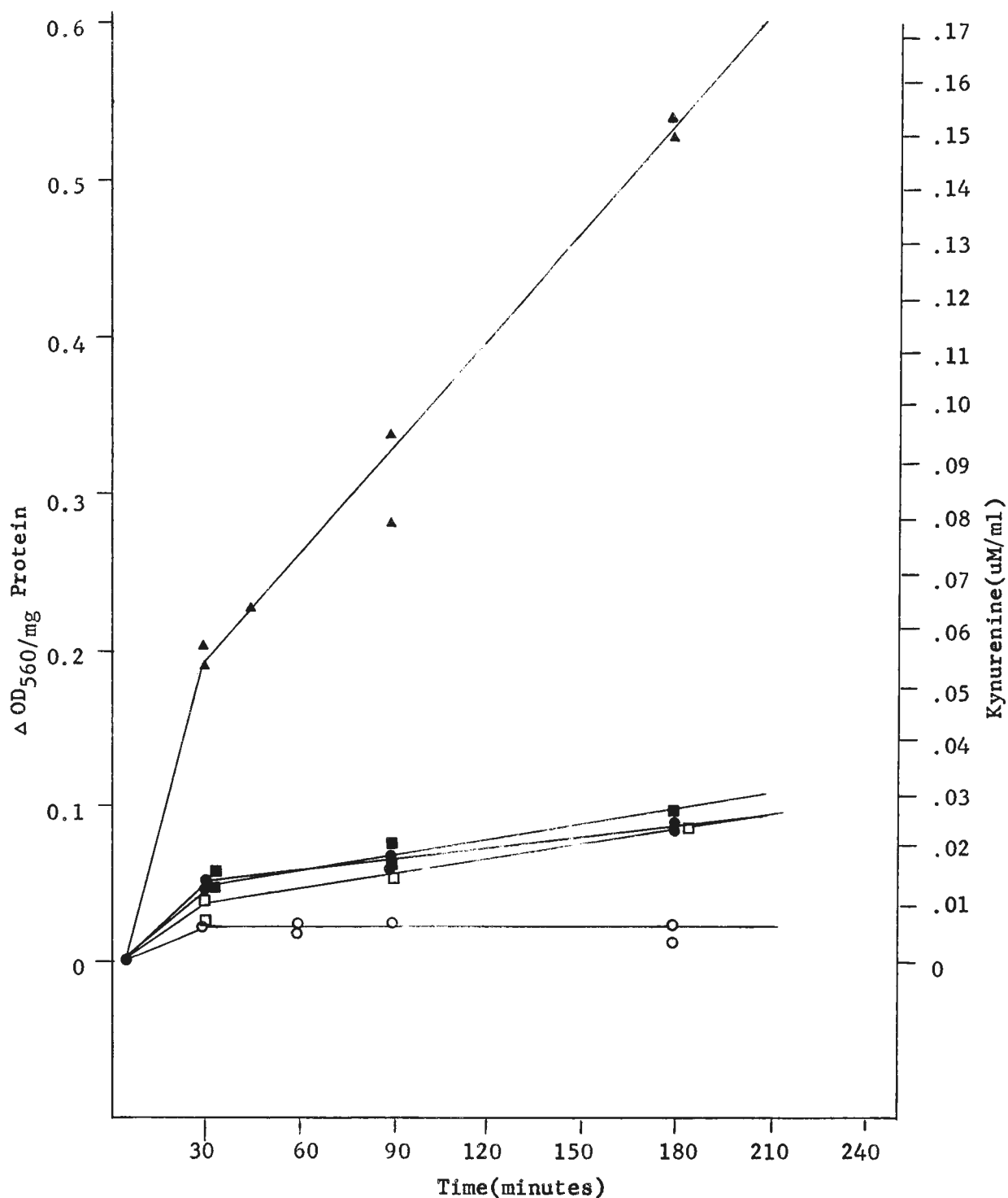


Figure 6. Stimulation of *in vitro* tryptophan pyrrolase synthesis by Canton-s and v^{36f} RNA: Ascorbate incubation, Bratton-Marshall assay.

ENZYME		INCUBATION	
(●-●)	PSS + Canton-s RNA	complete	
(○-○)	PSS + Canton-s RNA	without tryptophan	
(□-□)	PSS + tris buffer	complete	
(■-■)	PSS + v^{36f} RNA	complete	
(▲-▲)	Canton-s native	complete	

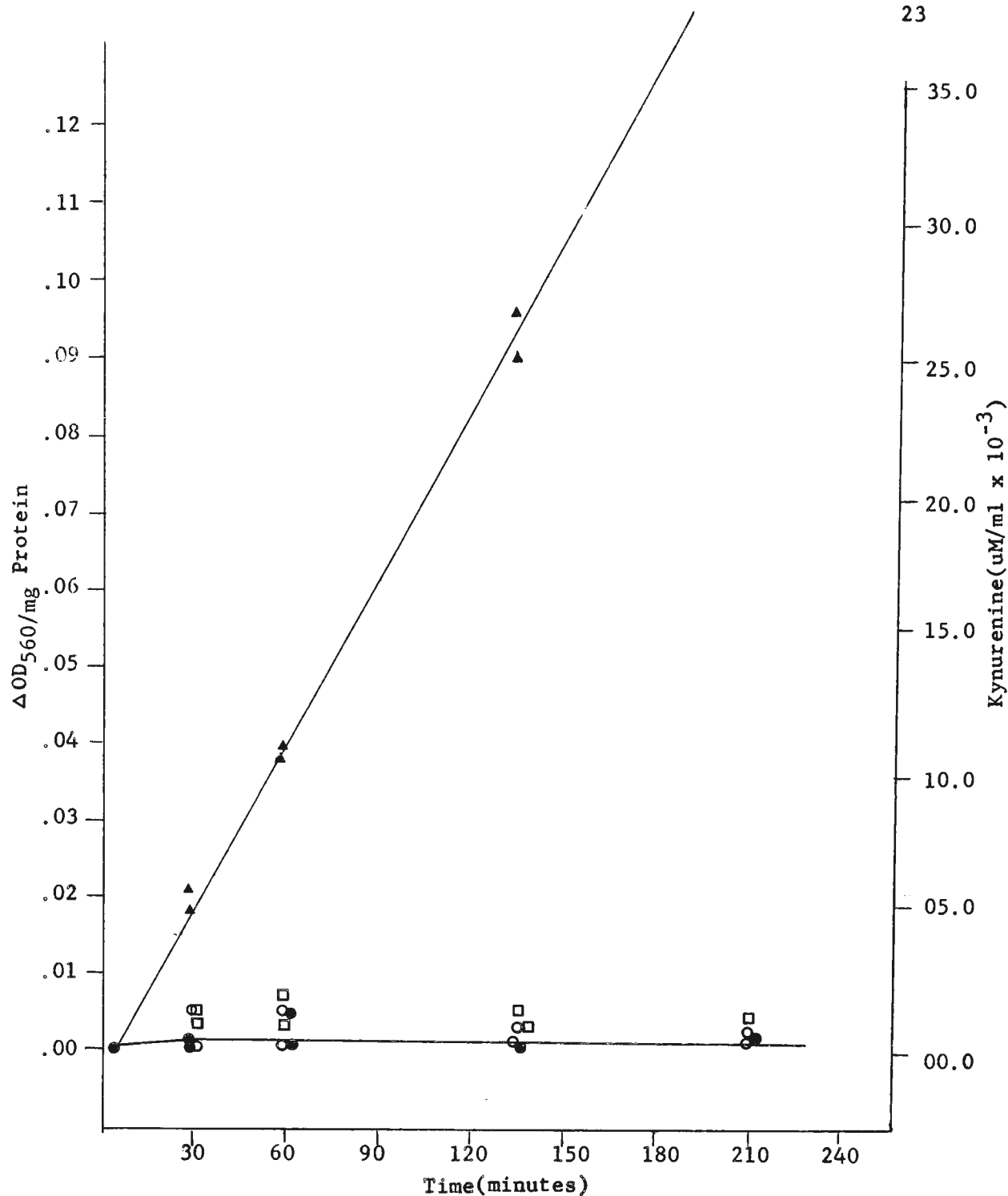


Figure 7. Stimulation of *in vitro* tryptophan pyrrolase synthesis by Canton-s and $\gamma^{36}\text{P}$ RNA: Mercaptoethanol incubation, Bratton-Marshall assay.

ENZYME		INCUBATION	
(o-o)	PSS + Canton-s RNA	complete	
(o-o)	PSS + Canton-s RNA	without tryptophan	
(-)	PSS + tris buffer	complete	
(-)	Canton-s native	complete	

Bratton-Marshall test shows no increase in activity by the addition of either Canton-s or \underline{v}^{36f} RNA. This also suggests the lack of tryptophan pyrrolase synthesis. The fourth criterion appears on the surface to be demonstrated by both assays. However, as was pointed out before, both assays detected a tryptophan dependent increase in "activity" occurring in the ascorbate incubation that was completely devoid of any enzyme. Since neither assay has the capacity to distinguish between the true activity of tryptophan pyrrolase and the "activity" seemingly inherent in the enzyme incubation system, it is impossible to conclude that the absence of a proper substrate, i.e., tryptophan, was the reason for the lack of activity observed in the no-tryptophan controls.

Validity of the Assays

The appearance in the ascorbate incubation system of a nonenzymatic reaction positive to both assays and the apparent discrepancies obtained between the two assay systems made it necessary to examine the validity of the enzyme incubation systems and of both assays used.

The response of both assays to kynurenine is shown in Figs 8 and 9. Each assay exhibits the expected linear response. Figs 10 and 11 show the effects of the protein synthesizing system on the assays. In both cases the magnitude of the curves show a positive shift; but the slopes remain unchanged, indicating no deleterious effect on either assay by the components of the protein synthesizing system.

The components of the enzyme incubation system were found to have a serious effect on both assays. Figs 12 and 13 show a direct adsorption positive, tryptophan-dependent reaction occurring in the ascorbate

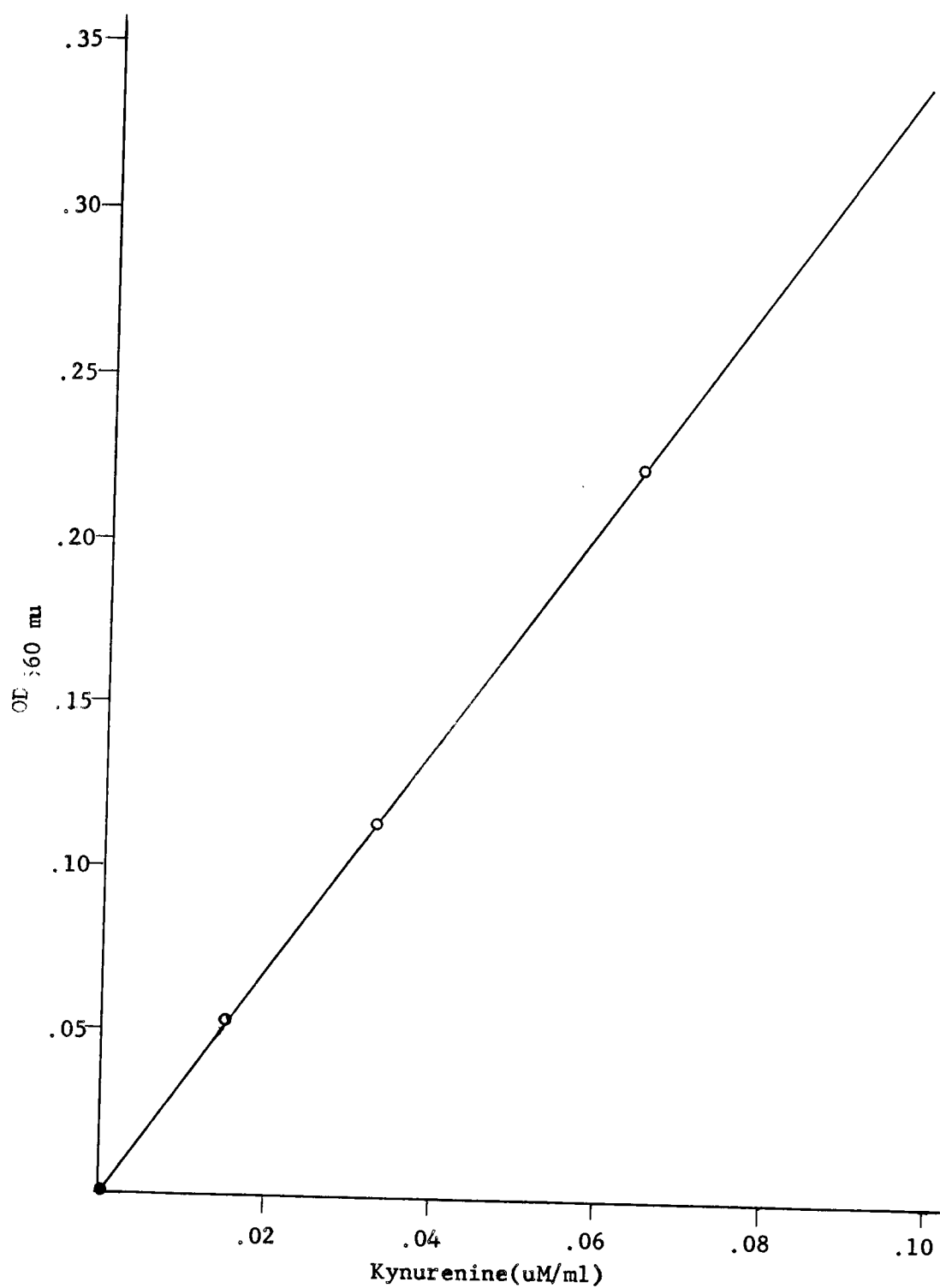


Figure 8. Kynurenine standard curve: Bratton-Marshall assay

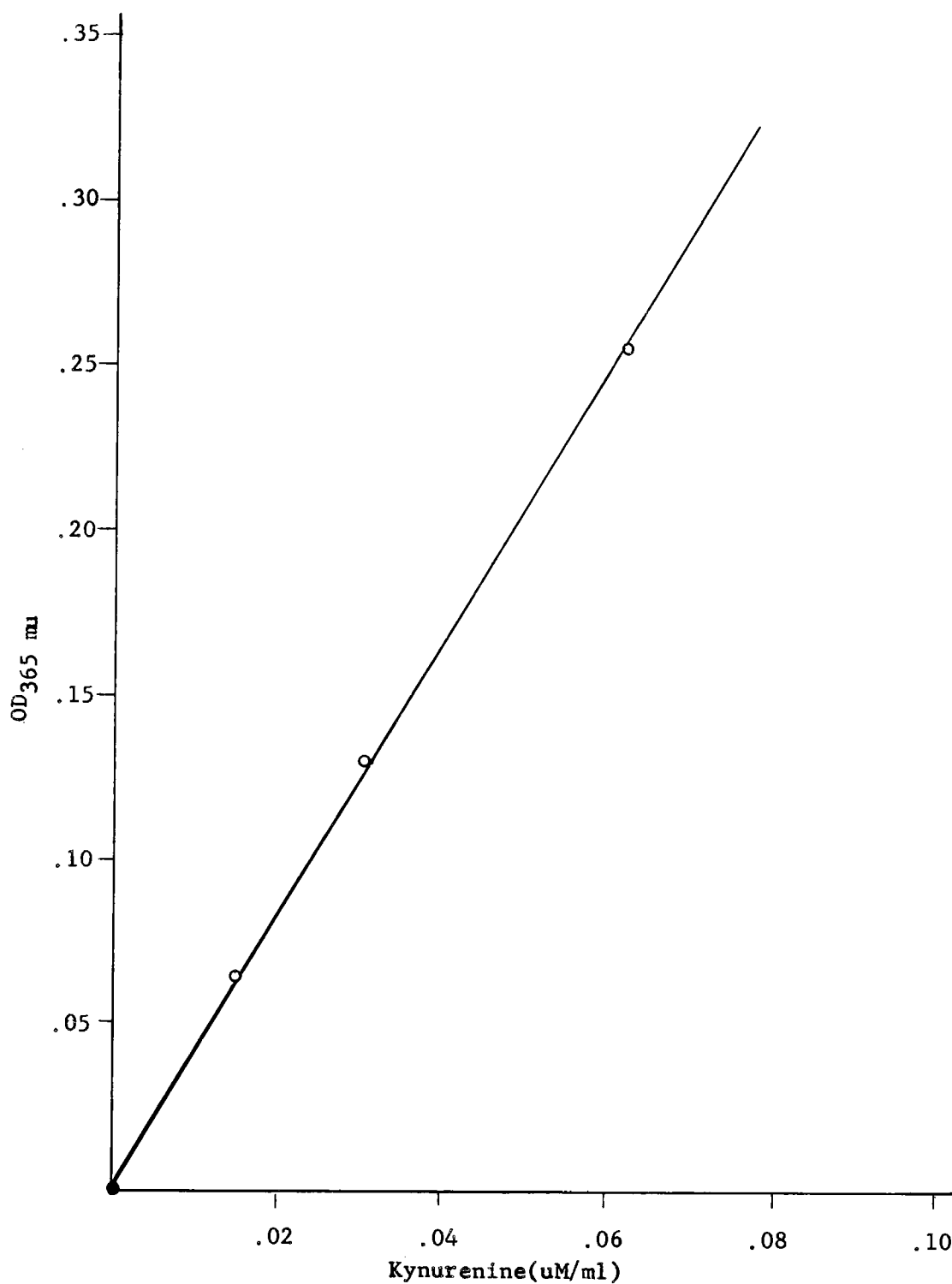


Figure 9. Kynurenine standard curve: Direct adsorption assay.

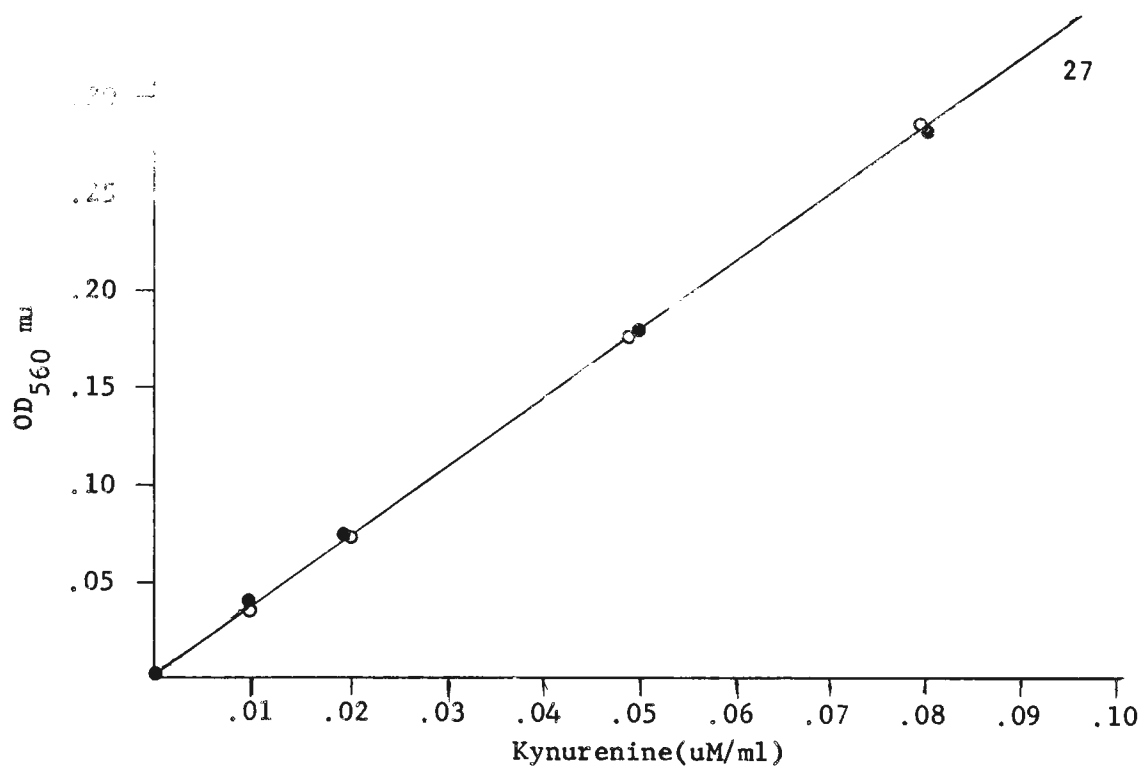


Figure 10. Effect of the PSS on the Bratton-Marshall assay.
 (o-o) Kynurenine standard curve; (●-●) Kynurenine standard curve in the presence of the PSS.

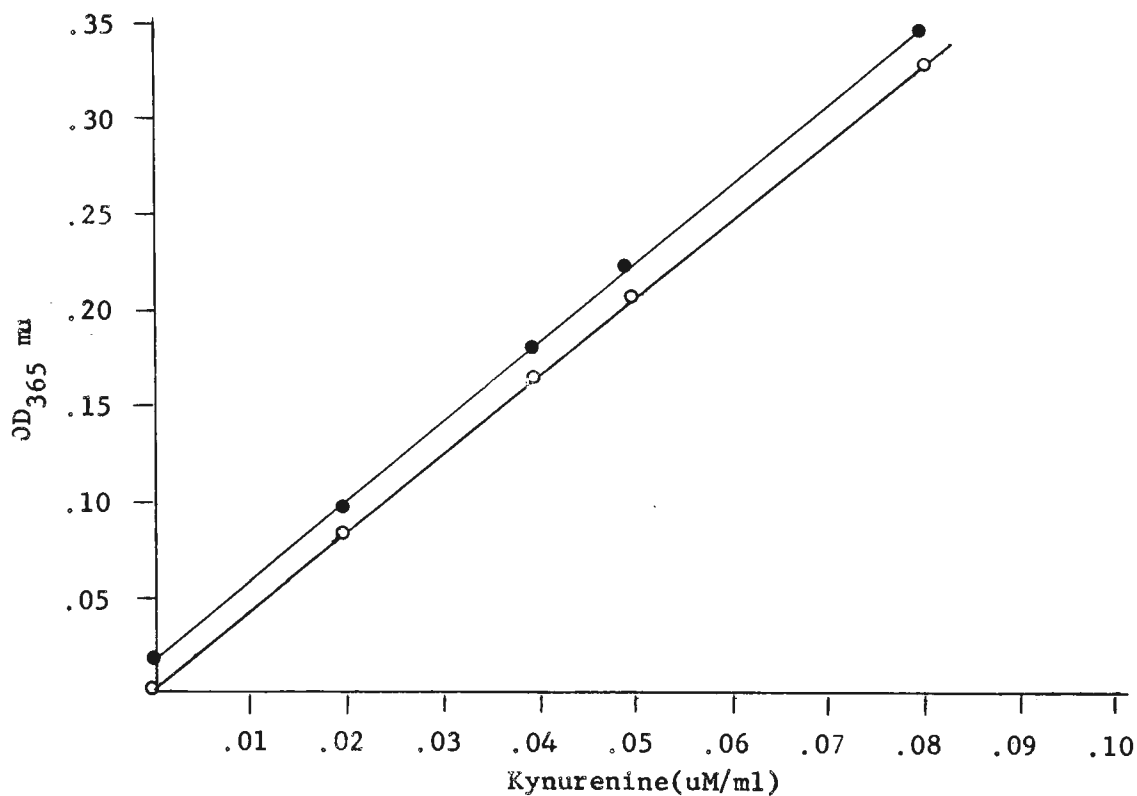


Figure 11. Effect of the PSS on the direct adsorption assay.
 (o-o) Kynurenine standard curve; (●-●) Kynurenine standard curve in the presence of the PSS.

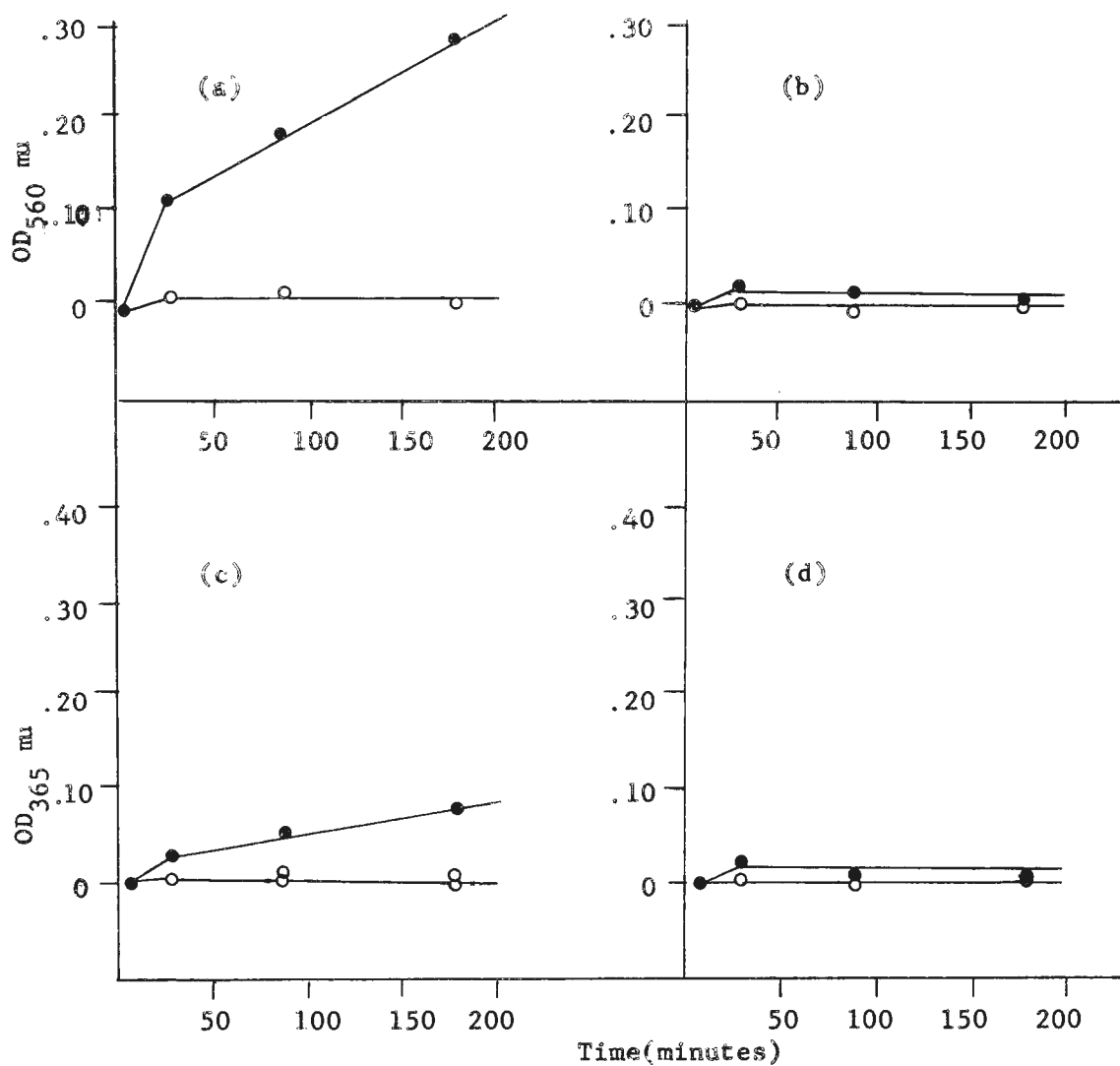


Figure 12. Assay of the ascorbate and 2-mercaptoethanol enzyme incubation systems by direct adsorption and the Bratton-Marshall test: (●-●) complete incubation system; (○-○) incubation system without tryptophan.
 12a. ascorbate incubation, direct adsorption assay
 12b. mercaptoethanol incubation, direct adsorption assay
 12c. ascorbate incubation, Bratton-Marshall assay
 12d. mercaptoethanol incubation, Bratton-Marshall assay

containing incubation system, but which disappears when the 2-mercaptoethanol incubation system (Kaufman, 1962) is used. The reaction is also Bratton-Marshall positive (Figs 14 and 15), and this increase is likewise eliminated by the use of the 2-mercaptoethanol system. The Canton-s RNA stimulated protein synthesizing system, when incubated in the 2-mercaptoethanol system, did not produce the increase in tryptophan pyrrolase "activity" as observed in the ascorbate system (Fig 7). These results show that neither the ascorbate containing enzyme incubation system nor the direct adsorption assay can be used reliably in the detection of tryptophan pyrrolase activity.

DISCUSSION

In attempting to repeat Morrison's results (Morrison, 1964), extreme care was taken to assure that all specified experimental conditions were met. The results shown in Fig 5 substantiate that the phenomena observed by Morrison are repeatable. In this sense the work is quite valid. Morrison's suggestion of in vitro synthesis of tryptophan pyrrolase was based on the following observations: Using a direct adsorption assay for kynurenine, it was found that certain unknown nondialyzable materials with adsorption maxima at 257 m μ which had been extracted from *Drosophila* homogenates produced an increase in OD 365 m μ when used in a cell-free protein synthesizing system. This increase was greater than that produced by a similar protein synthesizing system to which no extract had been added. This increase in OD demonstrated a dependence on the energy components of the protein synthesizing system and was sensitive to the presence of chloramphenicol, puromycin, RNase, and "sometimes" to DNase. The response of the system, based on these criteria, was used to support the hypothesis that the material used to stimulate the protein synthesizing system did, indeed, contain a tryptophan pyrrolase mRNA which was responsible for the in vitro coding of tryptophan pyrrolase.

The satisfaction of these criteria is generally accepted as being demonstrative of protein synthesis. However, the validity of their use depends entirely upon the method used for assaying the protein being synthesized. The assay for the synthesis of an enzymatic protein requires an enzyme incubation system in which the protein is allowed to catalyze a specific reaction. This reaction can then be assayed by

monitoring either the disappearance of the substrate or the appearance of the product. Two enzyme incubations have been used in the past for tryptophan pyrrolase. One utilizes ascorbate as the reducing agent (Kaufman, 1962), the other uses 2-mercaptoethanol as the reducing agent (Marzluf, 1965a). Marzluf has shown that the one utilizing ascorbate produces a nonenzymatic side reaction between tryptophan and ascorbate which yields a Bratton-Marshall positive product. The ascorbate system was used by Morrison (1964) and although he failed to recognize the tryptophan-ascorbate reaction, Figs 5, 12, and 14 show that the reaction is, indeed, present in the system he used. Failure to recognize this reaction has an obvious effect on the correct interpretation of tryptophan pyrrolase activity.

The criteria used by Morrison to detect in vitro protein synthesis must be re-examined in the light of their effect on the tryptophan-ascorbate reaction. Since the details of the reaction are unknown, the effects of chloramphenicol, puromycin, RNase, DNase, and of the energy system components on the reaction remain unknown. Since both the product of the tryptophan pyrrolase reaction and the product of the tryptophan-ascorbate reaction are read by both the direct adsorption and Bratton-Marshall assays, the effects of the tests used to determine RNA-dependent protein synthesis should also be studied in the light of their affect on the tryptophan-ascorbate side reaction before any valid conclusions can be drawn as a result of using the ascorbate incubation system. The use of the 2-mercaptoethanol incubation system, however, eliminates completely the nonenzymatic reaction and thus produces nothing to interfere with the correct assay of

kynurenine.

The use of a direct adsorption assay for quantitative determination of small amounts of material has been widely used. However, the determination of kynurenine in this system must be done in the presence of a complex, heterogeneous mixture of compounds including the components of the enzyme incubation system and the protein synthesizing system, which includes the energy generating system and all of the known and unknown components of the cell-free preparation. The use of a more specific assay would be desirable.

The Bratton-Marshall test, besides being more specific, is also three times more sensitive than direct adsorption. The effects of the protein synthesizing system and the mercaptoethanol incubation system on the Bratton-Marshall, as shown in Figures 10 and 14, are negligible. This assay, therefore, is superior to the direct adsorption assay.

The results of this study present a threefold argument against the interpretation of in vitro synthesis of tryptophan pyrrolase based on the observations reported by Morrison (1964). (1) ψ^{36f} RNA stimulates the same increase in tryptophan pyrrolase "activity" when added to a protein synthesizing system as does Canton-s RNA. Since ψ^{36f} RNA should not contain a tryptophan pyrrolase messenger the "activity" monitored by the direct adsorption assay cannot be due to tryptophan pyrrolase activity. (2) The same system which produces "activity" as monitored by direct adsorption shows no activity when assayed by the Bratton-Marshall. Since the Bratton-Marshall is (a) more specific than direct adsorption, (b) three times more sensitive than direct adsorption, and (c) is not interfered with by any of the components present in the

assay material, it can be concluded that the "activity" assayed by direct adsorption was not a result of tryptophan pyrrolase activity.

(3) The use of an ascorbate containing enzyme incubation system which was found to produce a tryptophan-ascorbate, nonenzymatic reaction positive to both assays, makes the interpretation of "activity" ambiguous. However, if the same protein synthesizing systems which show activity in the ascorbate incubation system are incubated in a 2-mercaptoethanol incubation system, no tryptophan pyrrolase activity is detected. Thus, it is concluded that although the observations reported by Morrison (1964) are valid, the interpretation that they demonstrate the in vitro synthesis of tryptophan pyrrolase is incorrect.

SUMMARY

A report (Morrison, 1964) of the in vitro synthesis of *Drosophila* tryptophan pyrrolase has been critically studied. Morrison's suggestion of the in vitro synthesis of tryptophan pyrrolase was based on the following observations: Using a direct adsorption assay for kynurenine, it was found that certain unknown nondialyzable materials extracted from *Drosophila* homogenates produced an increase in OD 365 mμ when used to stimulate a cell-free protein synthesizing system. This increase was greater than that produced by a similar protein synthesizing system to which no extract had been added and demonstrated a seeming dependence on an energy generating system.

The results of this study present a threefold argument against the interpretation of in vitro synthesis of tryptophan pyrrolase based on the observations reported by Morrison (1964). (1) RNA from a null-allele, v^{36f}, stimulates the same increase in tryptophan pyrrolase "activity" in a protein synthesizing system as does Canton-s RNA. (2) The Bratton-Marshall test, which has been shown to be a valid assay for kynurenine, does not assay any tryptophan pyrrolase activity in systems showing "activity" when assayed by the less specific direct adsorption method used by Morrison. (3) The ascorbate containing incubation system used by Morrison was found to produce a tryptophan-ascorbate, nonenzymatic reaction positive to both the direct adsorption and Bratton-Marshall assays. This side reaction is eliminated by the use of 2-mercaptoethanol in the place of ascorbate. If the same protein synthesizing systems which show activity when incubated in the ascorbate system are incubated in the 2-mercaptoethanol system,

no tryptophan pyrrolase activity is detected. Thus, it is concluded that although the observations reported by Morrison (1964) are valid, the interpretation that they demonstrate the in vitro synthesis of tryptophan pyrrolase is incorrect.

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